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RESEARCH PAPER

# The cadmium-tolerant pea (*Pisum sativum* L.) mutant SGECd$^t$ is more sensitive to mercury: assessing plant water relations

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## Abstract

**Heavy metals have multiple effects on plant growth and physiology, including perturbation of plant water status. These effects were assessed by exposing the unique Cd-tolerant and Cd-accumulating pea (*Pisum sativum* L.) mutant SGECd$^t$ and its wild-type (WT) line SGE to either cadmium (1, 4 μM $CdCl_2$) or mercury (0.5, 1, 2 μM $HgCl_2$) in hydroponic culture for 12 days. When exposed to Cd, SGECd$^t$ accumulated more Cd in roots, xylem sap, and shoot, and had considerably more biomass than WT plants. WT plants lost circa 0.2 MPa turgor when grown in 4 μM $CdCl_2$, despite massive decreases in whole-plant transpiration rate and stomatal conductance. In contrast, root Hg accumulation was similar in both genotypes, but WT plants accumulated more Hg in leaves and had a higher stomatal conductance, and root and shoot biomass compared with SGECd$^t$. Shoot excision resulted in greater root-pressure induced xylem exudation of SGECd$^t$ in the absence of Cd or Hg and following Cd exposure, whereas the opposite response or no genotypic differences occurred following Hg exposure. Exposing plants that had not been treated with metal to 50 μM $CdCl_2$ for 1 h increased root xylem exudation of WT, whereas 50 μM $HgCl_2$ inhibited and eliminated genotypic differences in root xylem exudation, suggesting differences between WT and SGECd$^t$ plants in aquaporin function. Thus, root water transport might be involved in mechanisms of increased tolerance and accumulation of Cd in the SGECd$^t$ mutant. However, the lack of cross-tolerance to Cd and Hg stress in the mutant indicates metal-specific mechanisms related to plant adaptation.**

**Key words:** Aquaporin, cadmium, drought, mercury, pea, root sap flow, water deficit.

## Introduction

Plant water status is determined by complex and interdependent processes such as root water absorption, water transport to the aerial organs, and transpiration, predominantly through the stomata. Heavy metals (such as Cd, Hg, Pb, Zn, Cu, and Ni) are widespread soil pollutants that are toxic to plants and can negatively affect all the processes related to plant water status (Poschenrieder and Barceló, 1999) through root growth inhibition that reduces total soil moisture availability to plants, decreased xylem vessel size (Kasim, 2007; De Silva *et al.*, 2012) and number (Barceló *et al.*, 1988; De Silva *et al.*, 2012), decreased root and stem hydraulic conductivity (Barceló *et al.*, 1988; Maggio and Joly, 1995; Nedjimi and Daoud, 2009), decreased stomatal density and conductance (Baryla *et al.*, 2001), and inhibited activity of the molecular water channels aquaporins (AQPs), thereby reducing the permeability of water across the cell membrane (Tazawa *et al.*, 1996; Savage and Stroud, 2007; Postaire *et al.*, 2010). Negative effects on AQPs were observed mostly in plants treated with mercury (Hg); therefore this metal was often used as an inhibitor of AQPs. Nevertheless, not all AQPs are sensitive to Hg (Biela *et al.*, 1999; Aroca *et al.*, 2006; Verdoucq *et al.*, 2008) and some aquaporin genes can be up-regulated by treatment

with this metal (Tamas *et al.*, 2010; Frick *et al.*, 2013) including the pea AQP *PsPIP-2* (Beaudette *et al.*, 2007). Heavy-metal induced perturbation of aquaporin function may decrease both root and shoot hydraulic conductance, thereby decreasing leaf water potential and turgor, which may directly close the stomata. Decreased leaf and root water status caused by heavy metals can enhance biosynthesis of the phytohormone abscisic acid (ABA) (Poschenrieder *et al.*, 1989), which acts to maintain plant water status. Root ABA biosynthesis can enhance root hydraulic conductivity (Thompson *et al.* 2007) and increase root-to-shoot ABA signalling thereby promoting stomatal closure (Davies and Zhang 1991; Dodd 2005). Indeed, decreased stomatal conductance observed in Cd-treated plants (Becerril *et al.*, 1989; Poschenrieder *et al.*, 1989; Zhu *et al.*, 2005) could be due to increased ABA concentrations. Nevertheless, Cd-induced stomatal closure may be independent of ABA signalling as it also occurred in the ABA-insensitive *Arabidopsis thaliana* mutant *abi1-1* (Perfus-Barbeoch *et al.*, 2002). It was also proposed that stomatal closure may decrease heavy metal uptake from soil thereby decreasing metal accumulation *in vivo* and its toxic effects (Pascual *et al.*, 2004; Lefevre *et al.*, 2009; Disante *et al.*, 2014).

The unique Cd-tolerant and Cd-accumulating multicellular plant mutant SGECd$^t$ was found in pea (*Pisum sativum* L.) line SGE (Tsyganov *et al.*, 2007) and provides promising new genetic material to study the effects of heavy metals on plant water relations, and may be important for understanding responses of plants to combined stresses (e.g. metal toxicity and drought). SGECd$^t$ was obtained by chemical mutagenesis, and is characterized by a monogenic inheritance and a recessive phenotype, and physiological mechanisms allowing enhanced biomass production under cadmium stress. Biochemical studies showed that in the presence of toxic Cd (4 µM) ions, the mutant SGECd$^t$ had lower contents of non-protein thiols and free proline, and activities of catalase and peroxidase than wild-type (WT) plants. Although it had adequate nutrient status, it actively accumulated Cd in roots, leaves, and mesophyll protoplasts (Tsyganov *et al.*, 2007), suggesting a cadmium-insensitive phenotype. To date, when grown under optimal conditions the only difference found between these pea genotypes was a 35% increase in the concentration of oxidized glutathione in SGECd$^t$ mutant roots (Tsyganov *et al.*, 2007). However, it was concluded that Cd tolerance of SGECd$^t$ was not linked to glutathione and/or phytochelatin biosynthesis, as only marginal genotypic differences in their concentrations were found in Cd-treated plants (Tsyganov *et al.*, 2007). Experiments with reciprocally grafted SGECd$^t$ and WT plants showed the crucial role of the root in the increased Cd-tolerance and Cd-accumulation of SGECd$^t$ (Malkov *et al.*, 2007), but the impact of cadmium on plant water relations was not assessed in previous reports (Tsyganov *et al.* 2007; Malkov *et al.*, 2007).

The present report aimed to characterize water relations (root hydraulic conductance, stomatal conductance, xylem vessel anatomy) of the pea mutant SGECd$^t$ in the absence and presence of both toxic Cd and Hg to elucidate the mechanisms underlying the effects of cadmium on plant water status. Mercury was included in some experiments as it inhibits AQP activity. Measurements of leaf water relations, xylem heavy metal concentrations, and root-to-shoot ABA signalling aimed to distinguish alternative mechanisms of stomatal closure.

## Materials and methods

### *Plant culture*

Seeds of wild-type (WT) pea (*Pisum sativum* L.) line SGE and its chemically induced mutant SGECd$^t$ characterized by increased Cd tolerance and Cd accumulation (Tsyganov *et al.*, 2007) were surface sterilized and scarified by treatment with 98% $H_2SO_4$ for 30 min, rinsed carefully with tap water, and germinated on filter paper in Petri dishes for 3 d at 25 °C in the dark. Seedlings were transferred to plastic pots (two pots with 10 seeds per genotype and treatment) containing 1500 ml of nutrient solution, which contained (µM): $KH_2PO_4$, 400; $KNO_3$, 1200; $Ca(NO_3)_2$, 60; $MgSO_4$, 250; KCl, 300; $CaCl_2$, 60; KCl, 250; Fe-tartrate, 12; $H_3BO_3$, 2; $MnSO_4$, 1; $ZnSO_4$, 3; NaCl, 6; $Na_2MoO_4$, 0.06; $AlCl_3$, 1; $CoCl_2$, 0,06; $CuCl_2$, 0.06; KJ, 0.06; KBr, 0.06; $NiCl_2$, 0,06; pH, 5.5. Plants were cultivated for 12 d in a naturally lit greenhouse (additional artificial lighting of 200 µmol $m^{-2}$ $s^{-1}$, a 12 h photoperiod with minima/maxima temperatures of 18 °C/23 °C). In experiments with long exposure to heavy metals (12 d), one day after planting (DAP) the nutrient solution was supplemented with: (i) 1 µM or 4 µM $CdCl_2$; (ii) 0.5, 1, or 2 µM $HgCl_2$. In all experiments the nutrient solution was changed, and where necessary the supplements were added, at 5 and 9 DAP.

In experiments with short exposure to heavy metals (1 h), the plants were cultivated in nutrient solution without metals for 11 d. On the 12th day the nutrient solution was supplemented or not with 50 µM $CdCl_2$ or 50 µM $HgCl_2$ (mercury was used to inhibit AQPs), the plants were treated for 1 h and roots were briefly washed with deionized water for 20 s. Then the plants from each metal treatment were transferred to pots with fresh nutrient solution and xylem sap allowed to exude from de-topped roots as described below.

### *Anatomical and physiological measurements*

To measure whole-plant transpiration rate on the day before harvest, pots were weighed at the beginning, middle, and end of the photoperiod and the time elapsed between weighing noted. Pots with and without plants were weighed to correct for evaporation from the surface of the nutrient solution and transpiration rate per plant was calculated. Stomatal conductance of young fully expanded leaves was measured with a transient time porometer (Model AP4, Delta-T Devices, Burwell UK) during the middle of the photoperiod. Before harvest, negative replicas of the lower leaf surfaces were taken using the Xantopren VL Plus (Heraeus Kulzer GmbH, Germany) and transferred as positive replicas to nail polish to measure stomatal density. At harvest, cross sections of roots (2–3 cm from cotyledons) were prepared and stained with toluidine blue (water solution containing 0.05 mg $ml^{-1}$ toluidine blue, 0.30 mg $ml^{-1}$ Na-benzoate and 0.25 mg $ml^{-1}$ benzoic acid) for 1 min to visualise xylem and phloem tissues in roots. Root cross sections and epidermal imprints were inspected and images were taken using a light microscope AxioVertA1 (Carl Zeiss, USA). Leaf images were analysed to count the number of stomata. Xylem and phloem area in roots was calculated using software OPTIMAS 6.1 (Optimas Corporation, Houston, USA).

### *Xylem sap exudation from de-topped roots*

Shoots were cut 2 cm above the cotyledons between 12.00 h and 15.00 h and cotton-filled 0.5 ml Eppendorf tubes (of defined weight) placed on the cut stumps and wrapped with PARAFILM® to prevent evaporative losses of sap. The pots were covered with aluminium

foil to avoid direct sunlight and sap was collected for 3 h. Then tubes were collected, weighed, and root sap flow rate ($J_v$) calculated per plant (total $J_v$) or per 1 mm$^2$ of xylem area (specific $J_v$).

### *Shoot xylem sap collection*

Shoot water potential was measured using a Scholander-type pressure chamber (Plant Moisture Systems, Santa Barbara, CA, USA). Xylem sap was then collected for 3 min from detached whole shoots at 0.5 MPa above balancing pressure (to gain enough sap for further analysis), frozen in liquid nitrogen, and stored at –20 °C. ABA concentration in xylem sap was determined through radioimmunoassay with the monoclonal antibody MAC252 (Quarrie *et al.*, 1988).

### *Leaf water relations*

Leaf water potential was measured in 8 mm diameter discs punched from young fully expanded leaves at the top of the canopy. Discs were incubated for 3 h in thermocouple psychrometers (C-52 chambers, Wescor Inc., Logan, UT, USA) before voltages were read with a microvoltmeter (HR-33T, Wescor Inc., Logan, UT, USA). Discs were then removed from the chambers, wrapped in aluminium foil, frozen in liquid nitrogen, and then re-inserted into the psychrometers and allowed to incubate for 30 min before leaf osmotic potential was measured. Voltages were converted to water and osmotic potentials based on calibration curves (for each individual psychrometer) with salt solutions of known osmotic potential. Leaf turgor was calculated by subtracting osmotic potential from water potential.

### *Cd and Hg concentrations*

Before harvest, roots were soaked in 5 mM Pb-citrate solution (pH 11) for 10 min and washed in deionized water for desorption of apoplastically bound Cd or Hg and then dried at room temperature. Then roots and leaves were dried at room temperature, ground, and digested in a mixture of concentrated $HNO_3$ and 38% $H_2O_2$ at 70 °C. Concentrations of Cd or Hg in digested plant samples and in xylem sap were determined using atomic absorption spectrophotometers AA-7000 (Shimadzu, Japan) or Varian AA240 FS with flow vapour generation accessory VGA 77 (Agilent Technologies, USA), respectively.

### *Statistical analysis*

Two way ANOVA along with an LSD test to discriminate differences between means was applied using the software STATISTICA version 10 (StatSoft Inc., USA).

## Results

### *Effects of long-term cadmium exposure*

Although 1 µM and 4 µM $CdCl_2$ significantly inhibited root and shoot growth of both pea genotypes (Fig. 1), the SGECd$^t$ mutant was much more tolerant to Cd. At 4 µM $CdCl_2$ the growth of WT plants was markedly decreased compared with plants grown in the absence of Cd, whereas the growth of the SGECd$^t$ mutant was similar to that of WT plants exposed to 1 µM $CdCl_2$ (Fig. 1). Images of typical control and treated (4 µM $CdCl_2$) plants are presented (Fig. 2E, F, G, and H).

In the presence of 1 µM $CdCl_2$, no genotypic differences were found in root, leaf, or xylem cadmium concentrations (Table 1). In the presence of 4 µM $CdCl_2$, root, xylem sap, and leaf cadmium concentrations of the mutant SGECd$^t$ were increased by 39%, 2.1-fold, and 11-fold, respectively,

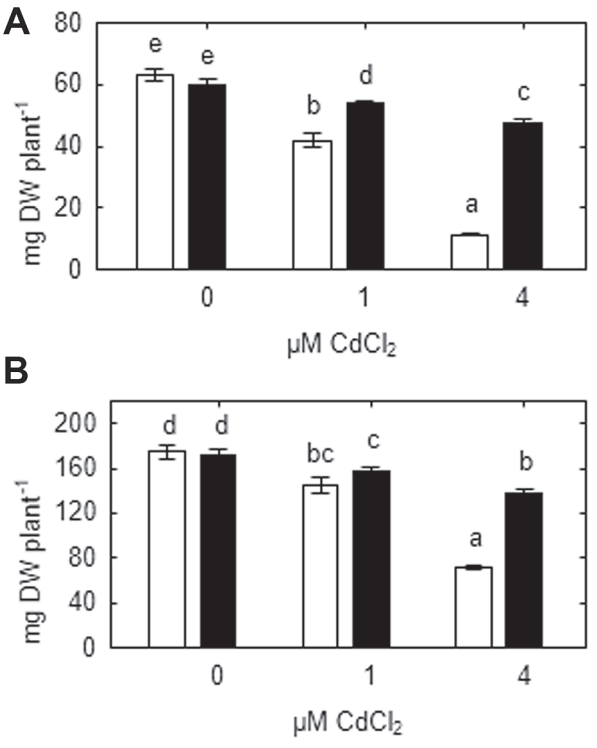


**Fig. 1.** Root (A) and shoot (B) biomass of SGE (open bars) and SGECd$^t$ (filled bars) pea genotypes grown at different cadmium concentrations. Different letters show significant differences between treatments (LSD test; $P<0.05$). Means of three experiments with at least five determinations each.

compared with WT plants. Xylem cadmium concentrations were similar to those in the nutrient solution, suggesting root-to-shoot Cd transport, particularly by the mutant plants treated with 4 µM $CdCl_2$ (Table 1). No Cd was detected in Cd-untreated plants (data not shown).

WT plants exposed to 4 µM $CdCl_2$ had decreased leaf water potential (Fig. 3A) and shoot water potential (Fig. 3B) compared with other genotype/treatment combinations. Increasing cadmium concentrations did not change leaf osmotic potential in the SGECd$^t$ mutant, but exposure to 1 and 4 µM $CdCl_2$ decreased it by 0.5–0.6 MPa in WT plants (Fig. 3C). These changes allowed WT plants to maintain turgor when exposed to 1 µM $CdCl_2$, but at 4 µM $CdCl_2$ leaf turgor of WT plants was significantly less than in SGECd$^t$ mutant plants (Fig. 3D).

Xylem sap exudation from de-topped roots of SGECd$^t$ plants was significantly higher than from WT plants in the absence of cadmium (by 48–66% according to the basis of expression), and was insensitive to cadmium concentration irrespective of whether data were expressed on a per plant, per root xylem area, or per root weight basis (Fig 4A–C). Treating WT plants with 1 and 4 µM $CdCl_2$ decreased sap exudation by 43% and 87%, respectively, on a whole-plant basis (Fig. 4A), and by 41% and 56%, respectively, on a per root xylem area basis (Fig. 4B) compared with untreated controls. However this effect was not observed when sap exudation was expressed per unit root weight (Fig. 4C).

Likewise, root xylem and phloem area of SGECd$^t$ plants was insensitive to cadmium concentration, but xylem and phloem area of WT plants treated with 4 µM $CdCl_2$ was

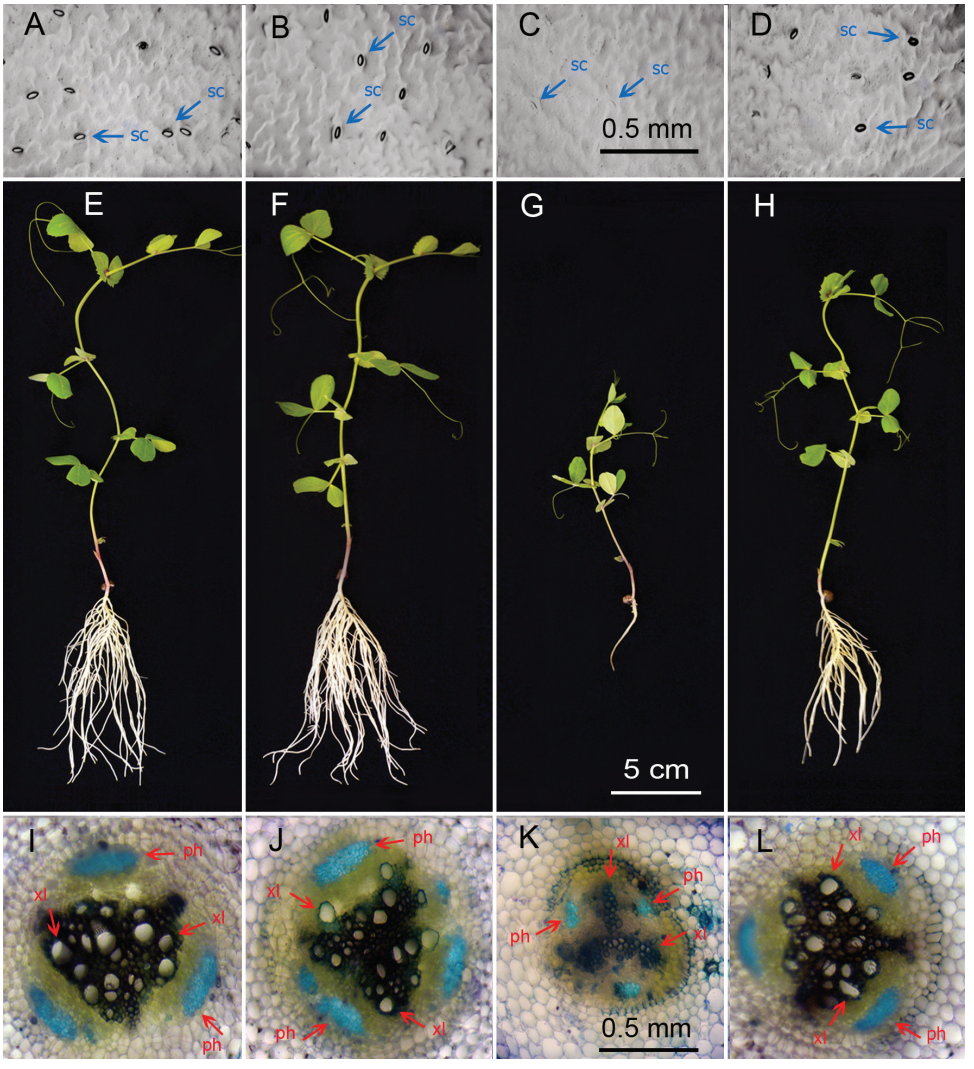


**Fig. 2.** Images of lower leaf surface (A–D) with stomatal complexes (sc) indicated, representative pea plants (E–H), and root cross sections (I–L) with xylem (xl) and phloem (ph) tissues indicated, from wild-type SGE plants untreated with cadmium (A, E, I), mutant SGECd$^t$ untreated with cadmium (B, F, J), wild type SGE plants treated with 4 µM $CdCl_2$ (C, G, K), and mutant SGECd$^t$ treated with 4 µM $CdCl_2$ (D, H, L).

**Table 1.** *Cadmium concentrations in pea plants grown in cadmium supplemented nutrient solution*

Data are means±SE of three experiments with at least four determinations. Different letters show significant differences within columns (LSD test, $P<0.05$).

| Genotype | Root, µg Cd $g^{-1}$ DW | Leaf, µg Cd $g^{-1}$ DW | Xylem sap, µM |
|---|---|---|---|
| **1 µM $CdCl_2$** | | | |
| SGE | 270±11 a | 20±2 ab | 1.3±0.08 a |
| SGECd$^t$ | 300±18 a | 30±5b | 0.9±0.05 a |
| **4 µM $CdCl_2$** | | | |
| SGE | 763±63 b | 11±1 a | 2.6±0.4 b |
| SGECd$^t$ | 1060±45 c | 120±7 c | 5.3±0.4 c |

2.5–3 times less than control treatments and SGECd$^t$ plants (Fig. 4D, E). Images of root cross sections from typical control and treated (4 µM $CdCl_2$) plants are presented (Fig. 2I–L).

Whole-plant transpiration and stomatal conductance of SGECd$^t$ plants was unaffected by 1 µM $CdCl_2$, but decreased by 26% and 50%, respectively, at 4 µM $CdCl_2$ (Fig 5A, B). In contrast, cadmium significantly decreased whole-plant transpiration of WT plants by 28% and 75% at 1 and 4 µM $CdCl_2$, respectively (Fig. 5A) and stomatal conductance by 52% and 95%, respectively. Differences in the percentage decrease between whole-plant transpiration and stomatal conductance probably reflect gradients in stomatal conductance according to the node at which the leaf is inserted, and the integrated effects of the treatments on stomatal conductance at all nodes affect whole-plant transpiration rate. Stomatal density did not vary with genotype or treatment (Fig. 5C). In control plants and those grown at 1 µM $CdCl_2$, xylem ABA concentration of WT and SGECd$^t$ plants did not significantly differ and was 19±10 nM and 11±5 nM, respectively (pooled across both treatments). Treatment with 4 µM $CdCl_2$ significantly increased xylem ABA concentration of WT plants by 30-fold, whereas the 3-fold increase observed in SGECd$^t$ was not statistically significant (Fig. 5D).

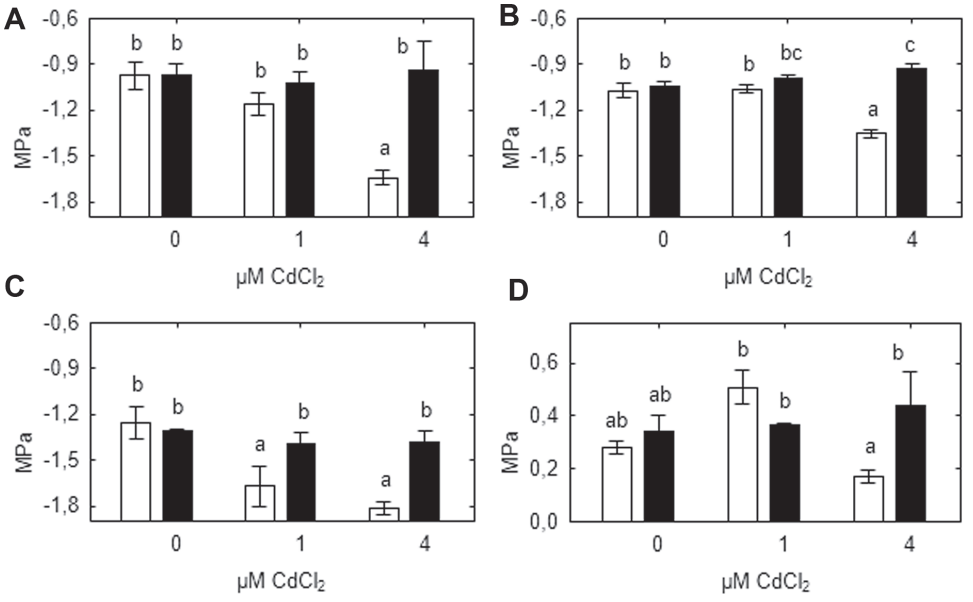


**Fig. 3.** Effect of cadmium on leaf water potential (A), shoot water potential (B), leaf osmotic potential (C), and turgor (D) of SGE (open bars) and SGECd$^t$ (filled bars) pea genotypes. Different letters show significant differences between treatments (LSD test; $P<0.05$). Means of three experiments with at least five determinations.

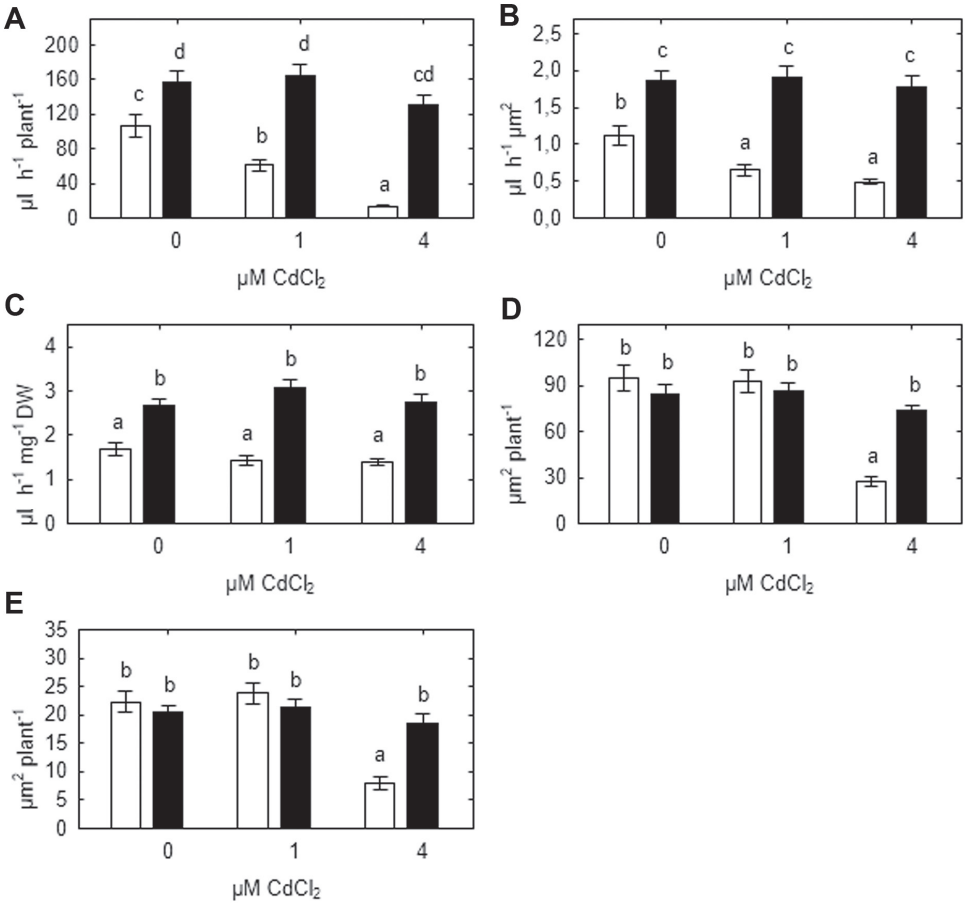


**Fig. 4.** Effect of cadmium on absolute root sap flow rate (A), and specific root sap flow rates expressed on a root xylem area (B) or root weight (C) basis, along with xylem (D) and phloem (E) area of SGE (open bars) and SGECd$^t$ (filled bars) pea genotypes. Different letters show significant differences between treatments (LSD test; $P<0.05$). Means of two experiments with at least three determinations.

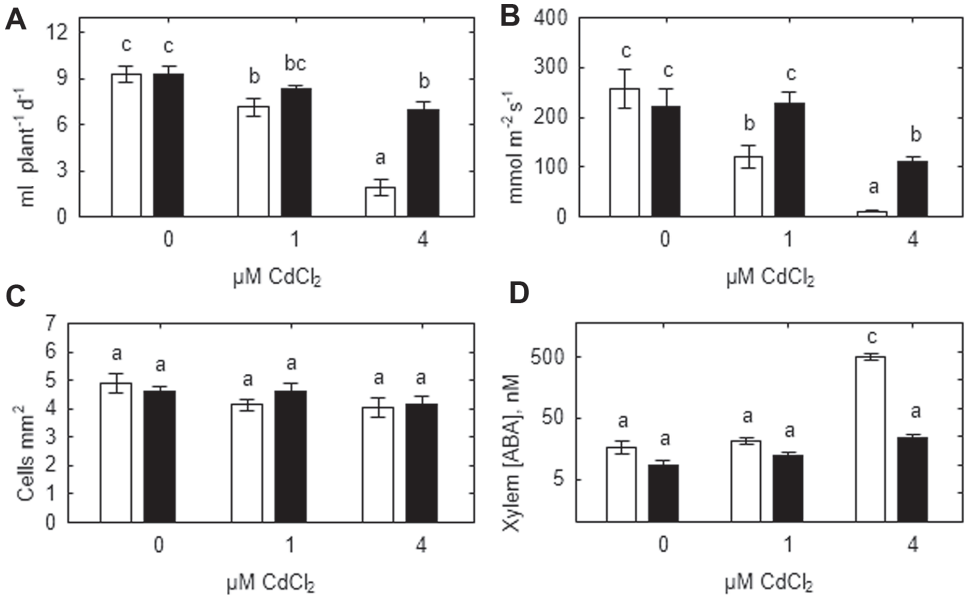


**Fig. 5.** Effect of cadmium on whole-plant transpiration (A), stomatal conductance (B), stomatal density (C), and xylem ABA concentration (D) of SGE (open bars) and SGECd$^t$ (filled bars) pea genotypes. Note the logarithmic scale on (D). Different letters show significant differences between treatments (LSD test; $P$<0.05). Means of two experiments with at least five determinations.

*Effects of long-term mercury exposure*

All mercury concentrations applied (0.5, 1, 2 μM $HgCl_2$) significantly inhibited root growth of both genotypes (Fig. 6A) and shoot growth of the SGECd$^t$ mutant even at 0.5 μM $HgCl_2$. At all mercury concentrations tested, growth of the SGECd$^t$ mutant was significantly less than the WT plants, suggesting lower Hg tolerance. Root mercury concentrations were similar in both genotypes in plants exposed to 1 μM or 2 μM $HgCl_2$ (Table 2). Generally, root mercury concentrations were three orders of magnitude higher than leaf mercury concentrations. Tissue mercury concentrations were not detected in Hg-untreated plants (data not shown) and not measured in plants treated with 0.5 μM $HgCl_2$.

In the absence of $HgCl_2$, xylem sap exudation of detopped WT plants was approximately halved compared with SGECd$^t$ mutant plants, irrespective of whether data were expressed on an absolute, xylem-area, or root weight basis (Fig. 7A–C), consistent with previous results (Fig. 3A–C). Treatment with 0.5 μM $HgCl_2$ increased xylem exudation of WT plants, thereby eliminating genotypic differences expressed on an absolute (Fig. 7A) or xylem-area specific (Fig. 7B) basis, but not on a root-weight specific basis (Fig. 7C). Treatment of SGECd$^t$ mutant plants with 0.5 μM $HgCl_2$ had no effect on absolute xylem exudation, but approximately doubled exudation on a root weight specific basis (Fig. 7C). Irrespective of the basis of expression, at 1 μM $HgCl_2$ xylem exudation of WT plants was significant higher than in SGECd$^t$ mutant plants but 2 μM $HgCl_2$ markedly inhibited root exudation in both pea genotypes and eliminated any genotypic differences.

Genotype and Hg concentration generally had no statistically significant effect on either xylem (Fig. 7D) or phloem (Fig. 7E) area, but 2 μM $HgCl_2$ significantly decreased xylem area of SGECd$^t$ plants by 50% (Fig. 7D). Stomatal conductance (gs) of WT plants was relatively insensitive to nutrient solution Hg concentration with 2 μM $HgCl_2$ decreasing gs by 19%, but 1 and 2 μM $HgCl_2$ decreased gs of SGECd$^t$ leaves by 43% and 74%, respectively (Fig. 7F).

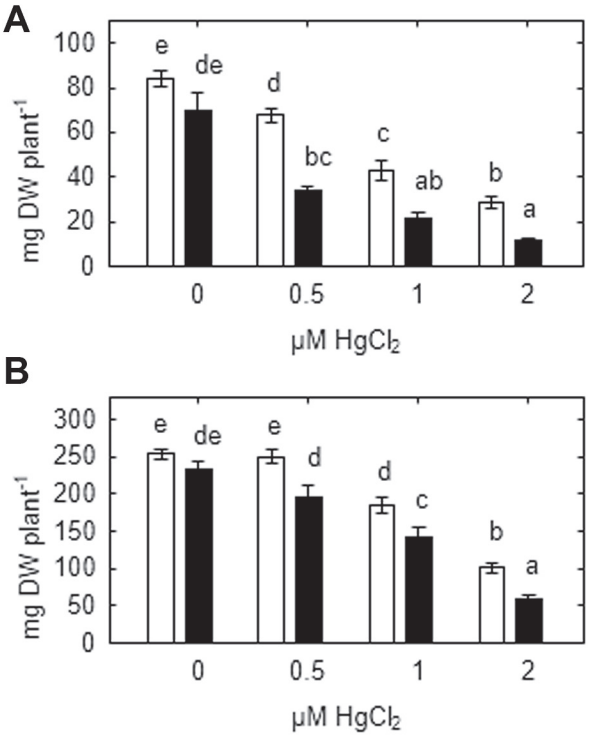


**Fig. 6.** Root (A) and shoot (B) biomass of SGE (open bars) and SGECd$^t$ (filled bars) pea genotypes grown at different mercury concentrations. Different letters show significant differences between treatments (LSD test; $P$<0.05). Means of two experiments with at least five determinations.

*Short-term effects of cadmium and mercury on xylem sap exudation*

As all plants used in this experiment were pre-cultivated for 12 d without cadmium or mercury, their biomass and root xylem area was similar (data not shown). Therefore only absolute root xylem exudation is presented (Fig. 8). As in previous experiments, xylem exudation of metal-untreated SGECd$^t$ plants was higher (84%) than WT plants. These genotypic differences were eliminated following short (1 h) exposure to cadmium (50 µM $CdCl_2$), which significantly increased xylem sap exudation in WT, but not SGECd$^t$ mutant plants. In contrast, treatment with mercury (50 µM $HgCl_2$) significantly reduced root xylem exudation of both genotypes and eliminated any genotypic differences between WT and mutant plants (Fig. 8).

**Table 2.** *Mercury concentrations in pea plants grown in mercury-supplemented nutrient solution*

Data are means±SE of one experiment with four determinations. Different letters show significant differences within columns (LSD test, $P<0.05$).

| Genotype | Root,µg Hg $g^{-1}$ DW | Leaf, µg Hg $g^{-1}$ DW |
|---|---|---|
| **1 µM $HgCl_2$** | | |
| SGE | 2370±240 ab | 2.4±0.1 a |
| SGECd$^t$ | 1832±215 a | 1.9±0.02 a |
| **2 µM $HgCl_2$** | | |
| SGE | 2777±138 bc | 10.3±0.9 b |
| SGECd$^t$ | 3047±262 c | 2.9±0.7 a |

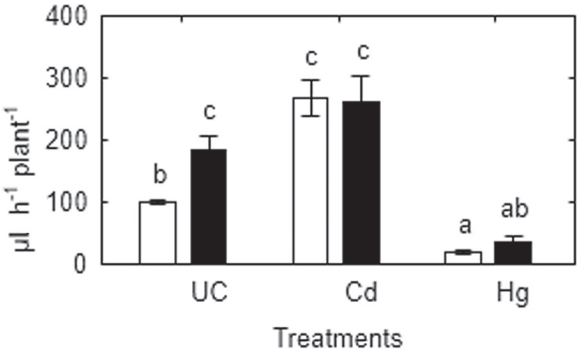


**Fig. 8.** Absolute root sap flow rate of SGE (open bars) and SGECd$^t$ (filled bars) pea genotypes after 1 h exposure to 50 µM $CdCl_2$ (Cd), 50 µM $HgCl_2$ (Hg) or untreated controls (UC). Different letters show significant differences between treatments (LSD test; $P<0.05$). Means of two experiments with at least four determinations.

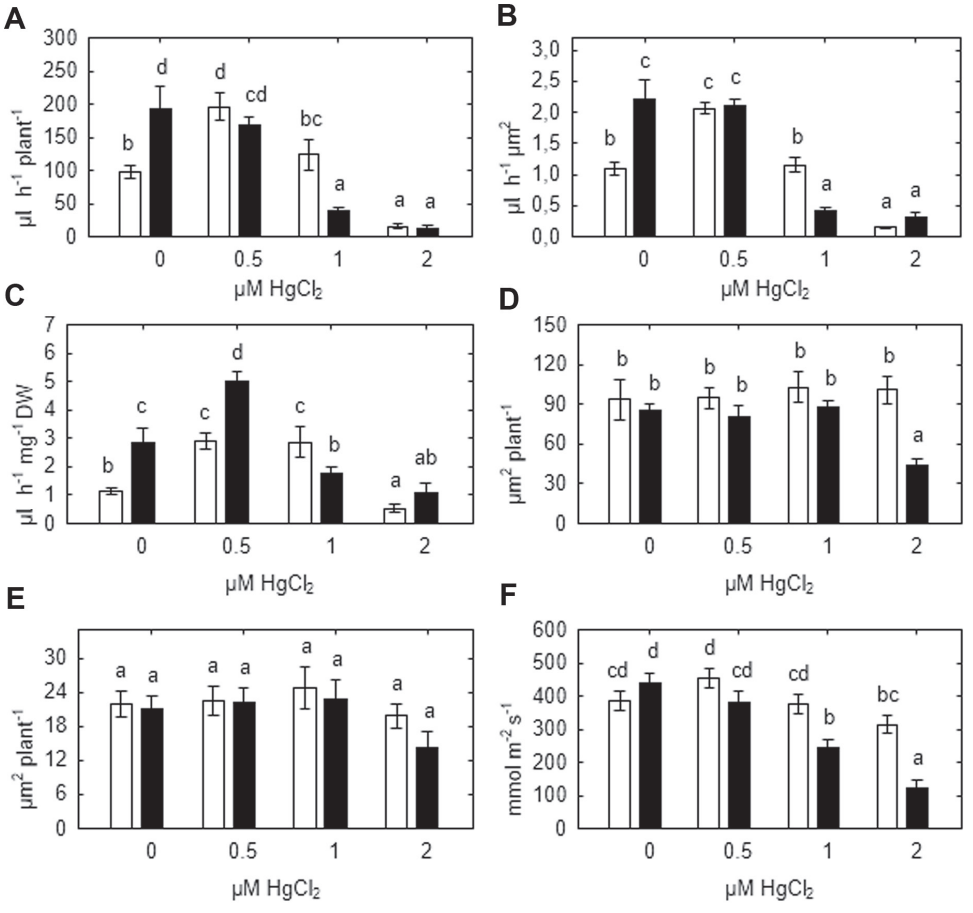


**Fig. 7.** Effect of mercury on absolute root sap flow rate (A), and specific root sap flow rates expressed on a root xylem area (B) or root weight (C) basis, along with xylem (D) and phloem (E) area and stomatal conductance (F) of SGE (open bars) and SGECd$^t$ (filled bars) pea genotypes. Different letters show significant differences between treatments (LSD test; $P<0.05$). Means of two experiments with at least five determinations.

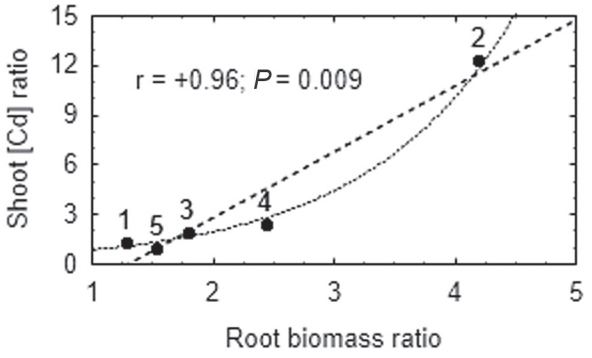


**Fig. 9.** Pooled data on relationship between the effect of cadmium on genotypic differences (SGECd$^t$/SGE ratio) in root biomass and shoot cadmium concentration. Black points show experimental data with cadmium concentrations in nutrient solution and reference: 1, 1 μM $CdCl_2$ (this study); 2, 4 μM $CdCl_2$ (this study); 3, 2.5 μM $CdCl_2$ (Tsyganov *et al.*, 2004); 4, 3 μM $CdCl_2$ (Tsyganov *et al.*, 2007); 5, 4 μM $CdCl_2$ (Malkov *et al.*, 2007). Dash and dotted lines show linear and exponential fitting, respectively.

## Discussion

### *Effects of Cd on water relations of the SGECd$^t$ mutant*

Consistent with previous work (Tsyganov *et al.*, 2007), the pea mutant SGECd$^t$ grew better and accumulated higher Cd concentrations in roots and leaves than WT plants. By compiling all available data where these genotypes were treated with different Cd concentrations, a positive correlation (r=+0.96, $P$=0.009, $n$=5) was detected between genotypic differences (SGECd$^t$/SGE ratio) in root biomass and in shoot Cd concentration (Fig. 9). This observation suggests that improved root growth (and probably root function) of the mutant compared with WT allows more active root-to-shoot xylem Cd transport and shoot Cd accumulation. Nevertheless, Cd-insensitivity allows the mutant to better maintain water (Fig. 3) and nutrient (Tsyganov *et al.*, 2007) status, resulting in better shoot growth. Taken together it may be proposed that the mutant has affected some gene(s) related to regulation of water uptake or/and transport in roots, and most probably the same gene(s) are involved in response of the plant to Cd toxicity. Previously, using reciprocally grafted plants, the crucial role of the root in the increased Cd-tolerance and Cd-accumulation of SGECd$^t$ was shown (Malkov *et al.*, 2007).

Cadmium dose-response curves allowed genotypic differences in physiology to be compared in plants of the same developmental stage, as effects of Cd on WT plants grown at 1 μM were similar those of mutant plants grown at 4 μM (Fig. 1). Previously, under similar growth conditions, in the presence of 3 μM $CdCl_2$, root and shoot (or leaf) Cd content of SGECd$^t$ was 19% and two times higher, respectively, than in the WT (Tsyganov *et al.*, 2007). Here, in the presence of 4 μM $CdCl_2$, such differences were much greater: 39% in roots and 11 times in leaves. When plants were treated with 1 μM $CdCl_2$, there were no significant genotypic differences in root, leaf, or xylem sap Cd concentrations, suggesting these effects depend on Cd concentrations in the nutrient solution. At 4 μM $CdCl_2$, xylem Cd concentration of the mutant was 2.1 times that of the WT and quite similar to that of the nutrient solution (5.3 μM). These results indicate that SGECd$^t$ actively transports Cd from root to shoot, and confirms significant root and shoot Cd accumulation within the SGECd$^t$ mutant, along with its high capacity to tolerate potentially toxic tissue Cd concentrations (Tsyganov *et al.*, 2007).

Maintaining high root water uptake capacity is very important for tolerance of plants to abiotic stresses caused by drought, flooding, salt, and low temperatures (Aroca *et al.*, 2012), but little is known about relationships between water uptake and heavy metal tolerance in different plant genotypes. An important finding was the greater absolute and specific (expressed per unit root weight or root xylem area) $J_v$ of the mutant SGECd$^t$ in the absence of toxic cadmium (Figs 4A–C, 7 A–C, and 8). Absolute and specific $J_v$ (expressed per unit root xylem area basis) significantly decreased with increasing Cd concentrations in WT plants, but not in the mutant SGECd$^t$ (Fig. 4A, B), and this correlated well with genotypic differences in plant growth (Fig. 1A). Exposing WT plants to 4 μM $CdCl_2$ for 12 d caused marked degradation of root xylem tissue, expressed as decreased lumen area (Figs 2K and 4D) and this could contribute to decreased $J_v$. Further measurements are required to determine whether these anatomical changes affect root hydraulic conductance *in vivo*. Indeed, Cd decreased root hydraulic conductivity of the newly found Cd-hyperaccumulating plant *Atriplex halimus* (Nedjimi and Daoud, 2009). Previously, Cd treatment (4.5 μM $CdCl_2$ for 21 d) reduced both the number and the size of xylem elements in bush bean stems (Barceló *et al.*, 1988) although no changes in diameter and number of metaxylem elements were found in roots of maize seedlings exposed to 100 μM $CdCl_2$ for 7 d (Maksimovic *et al.*, 2007). Although exposing maize seedlings to 5 μM $Cd(NO_3)_2$ for 10 d did not visibly alter root xylem tissues, significant lignification of endodermal and xylem cells was detected and interpreted as a defence mechanism preventing loading cadmium into xylem (Lux *et al.* 2011). The decreased area of phloem tissue in WT plants treated with 4 μM $CdCl_2$ (Fig. 2K) may also decrease the basipetal flow of photosynthates and thereby exacerbate negative effects of Cd on root growth and function. Paradoxically, expressing $J_v$ on a root weight basis indicated no effect of different Cd concentrations in either genotype (Fig. 4C), indicating close coordination of root biomass allocation and water transport in these genotypes. Irrespective of how $J_v$ is expressed, the effect of cadmium on shoot water relations may best indicate root function.

Treatment with 4 μM $CdCl_2$ decreased leaf and shoot water potentials and leaf osmotic potential of only WT plants (Fig. 3A, B). Similarly, Cd exposure decreased leaf osmotic potential and water content in leaves and roots of the Cd-treated halophyte species *Atriplex halimus* (Lefevre *et al.*, 2009). Although stomatal closure of plants exposed to 4 μM $CdCl_2$ acts to maintain leaf water status (as in drought-induced stomatal closure of pea where plants exposed to soil drying had a higher $\Psi_{leaf}$; Belimov *et al.*, 2009), the almost complete absence of root-pressure induced xylem exudation following long Cd-exposure (Fig. 4A) resulted in leaf water deficit (Fig. 3D). Cadmium-induced decreases in transpiration and stomatal conductance were repeatedly reported in different species, including legumes such as *Medicago sativa*

(Becerril *et al.*, 1989) and *Phaseolus vulgaris* (Poschenrieder *et al.*, 1989). Although long-term exposure to Cd (47 d in soil supplemented with 100 mg Cd $kg^{-1}$) can decrease stomatal density (Baryla *et al.*, 2001), in the experiments here there were no genotypic or Cd-dependent differences in stomatal density (Fig. 5C); thus transpiration rate was mostly controlled by stomatal apertures. Stomatal closure of WT plants exposed to 1 μM $CdCl_2$ in the absence of changes in leaf or shoot water potential (Fig. 3A, B) implied that root-sourced chemical signals were controlling leaf water status.

Stomatal closure has been correlated with increased foliar ABA concentrations in *Phaseolus vulgaris* (Poschenrieder *et al.*, 1989) and *Hordeum vulgare* (Hollenbach *et al.*, 1997). Similarly, decreased whole-plant transpiration (Fig. 5A) and stomatal conductance (Figs 2C and 5B) of WT plants treated with 4 μM $CdCl_2$ correlated with increased xylem ABA concentration (Fig. 5D), although it is not clear whether ABA was synthesized in the roots as a response to Cd toxicity or in the shoots as a result of shoot water deficit. Irrespective, xylem ABA concentrations were sufficiently high (0.5 μM in WT plants exposed to 4 μM $CdCl_2$) to induce stomatal closure, as feeding detached pea leaves similar ABA concentrations via the petiole approximately halved transpiration (Dodd *et al.*, 2008). An alternative explanation is that cadmium transported in the xylem (Table 1) enhances apoplastic Cd concentrations around the guard cells and closes stomata in an ABA-independent manner (Perfus-Barbeoch *et al.*, 2002). Further work to measure the sensitivity of detached leaf transpiration to xylem-supplied Cd will be needed to discriminate these competing hypotheses.

The data confirm that in the mutant there is greater Cd tolerance and accumulation of Cd, and demonstrate that root water uptake may be important in regulating these processes. Determining whether impaired shoot water relations inhibits shoot growth of WT plants exposed to high rhizospheric Cd concentration requires that these plants are grown at high humidity to obviate any change in leaf water status.

### *Effects of Hg on growth and water relations of the SGECd$^t$ mutant*

In contrast, the Cd tolerant SGECd$^t$ mutant was less tolerant to Hg, having lower root and shoot biomass, and stomatal conductance than WT plants under similar Hg treatments (Figs 6 and 7F). Decreased Hg tolerance of this mutant was associated with statistically equivalent root Hg concentrations, but substantially lower leaf Hg concentrations when plants were grown at 2 μM $HgCl_2$ (Table 2). These differences were opposite of that observed with Cd treatments, where simultaneously lower Cd tolerance and uptake were found in WT plants. In both cases genotypic differences in root metal concentrations were several times lower than differences in shoot metal concentrations, suggesting that root to shoot heavy metal transport is associated with the mutant phenotype and plays an important role in metal tolerance. The decreased Hg tolerance of SGECd$^t$ was accompanied by decreased stomatal conductance, which may decrease root-to-shoot Hg transport. Information about co-tolerance of plants to Cd and Hg is quite limited. A cadmium-tolerant cell line of tomato was similarly sensitive to Hg (Huang *et al.*, 1987). Comparing responses to Cd and Hg in *Zea mays* (Rellan-Alvarez *et al.*, 2006) and *Medicago sativa* (Ortega-Villasante *et al.*, 2005) revealed that biosynthesis of metal binding peptides phytochelatins was increased after treatments with Cd only, but not with Hg, suggesting different tolerance mechanisms to Cd and Hg. This study represents the first report of an inverse relationship between plant tolerance to these heavy metals, probably mediated by the same gene.

The molecular water channels AQPs, a numerous and multi-form family of proteins, can regulate root hydraulic conductance and water transport in various plant species, particularly under abiotic stress conditions (Maurel *et al.*, 2008, Aroca *et al.*, 2012). Although Cd had little effects on AQP activity of *Arabidopsis halleri*, it increased water permeability through the plasmalemma of individual leaf epidermal cells (Przedpelska *et al.*, 2008). Inserting the AQP gene *AtPIP2;1* from *A. thaliana* into the yeast *Pichia pastoris* showed that Cd but not Hg blocks this AQP (Verdoucq *et al.*, 2008). However, up-regulation of AQP genes in barley root tips was detected after treatments with either Cd or Hg (Tamas *et al.*, 2010). Here, a short treatment with a very high Cd concentration (50 μM $CdCl_2$) rapidly increased $J_v$ only in WT plants, indicating genotypic differences in response to different metal treatments (Fig. 8). Further experiments are required to determine whether increased $J_v$ of WT roots is due to up-regulation of AQP gene expression or some Cd-induced disturbances in regulation of these channels. In contrast to the conflicting effects of Cd on AQP gene expression and activity, treatment with millimolar Hg concentrations was repeatedly used to inhibit AQPs in plants (Maggio and Joly, 1995; Aroca *et al.*, 2001; Savage and Stroud, 2007; Postaire *et al.*, 2010), including pea (Beaudette *et al.*, 2007). Similarly, a short treatment with 50 μM $HgCl_2$ not only inhibited $J_v$, but also eliminated genotypic differences in this parameter, suggesting that AQP regulation differs in SGECd$^t$ mutant and WT plants and that AQPs may be involved in the increased $J_v$ of the mutant under optimal conditions (Fig. 8).

An unexpected result was that long exposure to relatively low Hg concentration (0.5 μM) significantly increased $J_v$ of WT pea to the level of the SGECd$^t$ mutant, without affecting root xylem and phloem areas (Fig. 7). Up-regulation of AQP genes in response to chronic Hg concentrations may be involved (Tamas *et al.* 2010). Similarly, increased root hydraulic conductance under relatively long exposure to a mild stresses such as chilling and oxidative stress (Aroca *et al.*, 2005) or drought and ABA treatment (Aroca *et al.*, 2006) was a result of adaptive responses in expression of AQP genes and AQP protein abundance. Thus further investigations of the role of AQPs in the water relations of the studied plant genotypes is needed to elucidate the mechanisms involved.

## Conclusion

When grown in Cd-supplemented solution, the Cd-tolerant and Cd-accumulating pea (*Pisum sativum* L.) mutant SGECd$^t$ had better water uptake and transport (moderated

by morphology of root vascular tissues, and diminished xylem ABA concentration, but higher stomatal conductance, leaf water and osmotic potentials, leaf turgor, and whole-plant transpiration) than the WT SGE line. The mutant also had higher root sap flow rate than WT plants in both the presence and absence of toxic Cd ions. These observations suggest that root water transport might be involved in mechanisms of increased tolerance and accumulation of Cd in SGECd$^t$, which has a Cd-insensitive phenotype. In contrast, SGECd$^t$ possessed decreased Hg-tolerance and foliar Hg-accumulation, and the negative effects of Hg on water relations parameters were more pronounced in the mutant. Such genotypic specificity in tolerance to different heavy metals has not been described previously and provides new evidence for the importance of water relations, particularly proper root function in water uptake and transport, in tolerance to and accumulation of heavy metals by plants.

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